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The antiproliferative effects of phenoxodiol are associated with inhibition of plasma membrane electron transport in tumour cell lines and primary immune cells

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ABSTRACT

Although the redox-active synthetic isoflavene, phenoxodiol, is in Phase 3 clinical trials for drug-resistant ovarian cancer, and in early stage clinical trials for prostate and cervical cancer, its primary molecular target is unknown. Nevertheless, phenoxodiol inhibits proliferation of many cancer cell lines and induces apoptosis by disrupting FLICE-inhibitory protein, FLIP, expression and by caspase-dependent and -independent degradation of the X-linked inhibitor of apoptosis, XIAP. In addition, phenoxodiol sensitizes drug-resistant tumour cells to anticancer drugs including paclitaxel, carboplatin and gemcitabine. Here, we investigate the effects of phenoxodiol on plasma membrane electron transport (PMET) and cell proliferation in human leukemic HL60 cells and mitochondrial gene knockout HL60ρ° cells that exhibit elevated PMET. Phenoxodiol inhibited PMET by both HL60 (IC_{50} 32 μM) and HL60ρ° (IC_{50} 70 μM) cells, and this was associated with inhibition of cell proliferation (IC_{50} of 2.8 and 6.7 μM, respectively), pan-caspase activation and apoptosis. Unexpectedly, phenoxodiol also inhibited PMET by activated murine splenic T cells (IC_{50} of 29 μM) as well as T cell proliferation (IC_{50} of 2.5 μM). In contrast, proliferation of WI-38 cells and HUVECs was only weakly affected by phenoxodiol. These results indicate that PMET may be a primary target for phenoxodiol in tumour cells and in activated T cells.

1. Introduction

The synthetic isoflavene, phenoxodiol [1], is in advanced clinical trials for the treatment of drug-resistant ovarian cancer and is in early stage trials for prostate and cervical cancer, both as a single agent and in chemo-sensitization settings. However, despite progression into clinical trials, the primary cellular target or targets of phenoxodiol remain elusive. Rather, the drug is often referred to as a multiple signal transduction regulator [2], a term consistent with its redox activity and multiple targets of the parent compound, genistein. Mechanistic studies have shown that phenoxodiol inhibits DNA topoisomerase II [1], and induces apoptosis in ovarian carcinoma [3,4] and melanoma cells [5]. Both caspase-dependent and -independent apoptosis have been described. With chemoresistant ovarian cancer cells, phenoxodiol induced early ubiquitination and degradation of the X-linked inhibitor of apoptosis, XIAP, via the proteosome [3,4], and disrupted expression of the FLICE-inhibitory protein, FLIP, through the Akt signal transduction pathway [4]. With other human cancer cell lines including head and neck and colon carcinoma, phenoxodiol induced caspase-independent apoptosis following G1 cell cycle arrest and this was associated with $p21^{WAF1}$ expression [6]. Induction of apoptosis by anticancer drugs is often a consequence of a primary site or sites of action physically distinct from the pro-apoptotic machinery

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rather than being a direct target. Therefore, understanding the primary molecular target and the linkage of this target to key components of apoptotic pathways is central to optimizing drug development and usage.

Fine control of the intracellular redox status of cells is critical for cell viability, growth and function. Perturbation of key redox couples such as the NADH/NAD^+ ratio, glutathione balance and membrane ubiquinone redox status and content can have profound effects on cells resulting in apoptosis [7,8]. Small redox-active amphipathic molecules such as phenoxodiol may disrupt essential redox cycling at many different levels within cells and their membranes. Because phenoxodiol is poorly water-soluble and requires the use of organic solvents, it will have a tendency to partition in membrane systems. This suggests that redox-active molecules in membranes may be primary targets for phenoxodiol action. Two main membrane systems orchestrate the bioenergetic status of cells. The inner mitochondrial membrane contains an electron transport system that is responsible for generating the membrane potential that drives most ATP production via oxidative phosphorylation. Ubiquinone is an essential redox-active mediator of mitochondrial electron transport (MET), shuttling electrons from Complexes I and II to Complex III. In the absence of mitochondrial electron transport, cells must rely on glycolysis to generate ATP. Increasing evidence indicates that the plasma membrane also contains an essential electron transport system that utilizes ubiquinone as a one electron acceptor and donor [9,10], and that this system may support glycolytic ATP production by oxidizing cytosolic NADH [11,12], a role usually attributed to lactate dehydrogenase (LDH). This suggests that plasma membrane electron transport (PMET) may be a primary site of action for the anticancer activity of redox-active lipophilic flavonoids such as phenoxodiol, catechins like epigallocatechin gallate, resveratrol and quinone-containing anticancer drugs such as doxorubicin.

Phenoxodiol was recently shown to bind with high affinity to a purified recombinant NADH-oxidase, compromising its ability to oxidize both NADH and ubiquinol and to catalyze protein disulfide–thiol interchange activity [13]. This protein is a truncated form of a tumour-specific cell surface NADH-oxidase, tNOX, thought to be involved in the transfer of electrons from intracellular NADH to an extracellular acceptor via plasma membrane ubiquinone [14,15]. These effects of phenoxodiol were shown to be associated with inhibition of HeLa cell enlargement and growth [13]. In contrast, activity of the constitutively-expressed cell surface NADH-oxidase, CNOX, present on both cancer and non-cancer cells was said to be unaffected by phenoxodiol.

In order to directly assess the effects of phenoxodiol on PMET, we have used mitochondrial gene knockout (ρ°) cells [11,16] in which bioactivity can be attributed to interaction with redox-sensitive systems other than MET, greatly simplifying interpretation of results. Using this system, we investigated the effects of phenoxodiol on PMET and cell proliferation in human leukemic HL60 cells and HL60ρ° cells, activated murine splenic T cells and primary human cells in culture. Phenoxodiol inhibited PMET and cell proliferation of both HL60 cells and activated primary splenic T cells to a similar extent, while HL60ρ° cells were less sensitive. These results support a primary mechanism of action of phenoxodiol that includes PMET and indicate that inhibition of PMET compromises proliferation of both HL60 cells and activated primary immune cells.

2. Materials and methods

2.1. Cells and cell culture

HL60 cells, originally from ATCC, were obtained from Dr. Graeme Findlay (University of Auckland, NZ); WI-38 at passage 7 were from Dr. David Brown (Novogen, Australia), and were expanded from passages 9–10 stocks; primary HUVEC cells at passages 3–4 were from Dr. Sara Gunningham (Christchurch School of Medicine and Health Science, University of Otago). All cell lines used were free of mycoplasma. The mitochondrial DNA-knockout cell line, HL60ρ°, was derived from its parental cell line, HL60, by culturing in the presence of ethidium bromide for 6–8 weeks [17] and lack of mitochondrial DNA verified by PCR and stable phenotype.

Cycling T cells were generated as follows: a single cell suspension of spleens from 8 to 10 weeks old C57BL/6J mice were generated by teasing through a 70 μM cell strainer using IMDM medium containing 5% (v/v) fetal bovine serum, 25 μg/mL penicillin, 25 μg/mL streptomycin and 0.5 μM 2β-mercaptoethanol. Splenocytes, 2×10^6 cells/mL, were stimulated with 0.1 μg/mL 145-2C11 anti-CD3 antibody and 20 μL/mL of 37.51.1 anti-CD28 hybridoma supernatant for 3 days. At this stage, FACS analysis showed that the T cell cultures contained approximately 70% CD8 T cells, 15% CD4 T cells, and 15% other cells (mainly B cells).

For cell surface oxidase assays, T cells were washed twice in HBSS before being used. For MTT assays, T cells were washed twice with complete IMDM medium and incubated with various concentrations of phenoxodiol in the presence of 100 U/mL of IL-2.

All cell lines were grown in RPMI-1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 5% (v/v) fetal bovine serum, 2 mM glutamate, 25 μg/mL penicillin, 25 μg/mL streptomycin, 50 μg/mL uridine and 1 mM pyruvate to densities of $(1–2) \times 10^6$ cells/mL (exponential stage), at 37 °C in a humidified incubator maintained at 5% CO_2.

2.2. Materials

2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) and 1-methoxyphenazine methylsulfate (1 mPMS) were purchased from Dojindo Laboratories (Kumamoto, Japan). Phenoxodiol was obtained from Novogen Inc. (NSW, Australia) and Doxorubicin hydrochloride was purchased from Faulding Pharmaceuticals (Warwickshire, UK). Mouse anti-human PE-labelled CD95 (Fas), propidium iodide (PI), fluorescein isothiocyanate (FITC)-labelled Annexin V (AV) and Annexin V binding buffer were from Pharmingen (Becton Dickinson, North Ryde, Australia). The antibody 145-2C11 (anti-CD3εγ/εδ) was purified from culture supernatant. Supernatant from cells transfected with recombinant mIL-2 was used as a source of IL-2. Supernatant from the 37.51.1 hybridoma cell